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Identification of the replication region of *Lactobacillus acidophilus* plasmid pLA103

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Abstract

The structure of the region necessary for replication of the plasmid pLA103 from *Lactobacillus acidophilus* TK8912 has been characterized. Sequence analysis revealed that the replication region contained an open reading frame (OrfA) encoding a 282-amino acid peptide preceded by a 22-bp tandem repeat sequence region. The predicted OrfA protein showed homology to the replication protein of a plasmid from *Pediococcus halophilus*. The plasmid containing the repeat sequence region preceding OrfA was able to replicate in the *Lactobacillus* host when provided with OrfA *in trans*, suggesting that the repeat sequence region contains the origin sequence essential for the pLA103 replication.

Keywords: *Lactobacillus acidophilus*; *Lactobacillus* plasmid; Plasmid replication; Plasmid vector

1. Introduction

The members of the genus *Lactobacillus* are well recognized as industrially important microorganisms in dairy fermentation. Many *Lactobacillus* strains harbor one or more plasmids of various sizes. Some industrially significant traits, including lactose fermentation and bacteriocin production, are often associated with the specific plasmids. The genetic determinants of bacteriocins have a great potential as the selectable marker for the construction of food-grade vectors aimed at genetic manipulation of actual dairy starter strains.

The plasmid pLA103, which is 14 kb, was originally isolated from *Lactobacillus acidophilus* TK8912, and was associated with bacteriocin production and host immunity [1]. In preliminary experiments, pLA103 has been shown to replicate and be maintained in several *Lactobacillus* hosts. The development of vectors using this plasmid would therefore be useful for the construction of genetically altered strains for experimental purposes and for industrial fermentations. Here, we have determined the nucleotide sequence and analysed the molecular structure of the region necessary for replication of the pLA103 plasmid.

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2. Materials and methods

2.1. Bacterial strains and media

L. acidophilus TK8912 harboring pLA103 was obtained from the laboratory collection [2]. *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) was used for cloning. *L. acidophilus* cultures were grown in MRS medium [3], and *E. coli* were cultured in Luria-Bertani medium [4].

2.2. Plasmid DNA preparation and manipulations

E. coli plasmids were isolated according to the method of Birnboim and Doly [5]. Plasmid DNA of *L. acidophilus* was prepared as described [2]. Restriction enzymes and DNA-modifying enzymes were used as recommended by suppliers. General cloning techniques were carried out as described by Sambrook et al. [4].

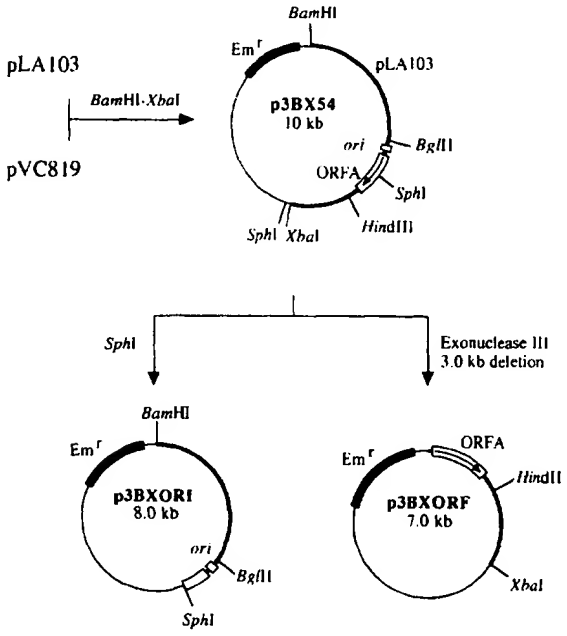

Fig. 1. Construction of p3BX54 and its derived plasmids. Symbols on p3BX54: the thick line represents the 5.4-kb *Bam*HI–*Xba*I fragment originating from pLA103; the thin line represents the DNA fragment originating from pVC819. Em^r and *ori* represent the erythromycin resistance gene and the putative origin site, respectively. p3BXORI is the mutant with a deletion of the *Sph*I–*Sph*I fragment of p3BX54, and p3BXORF is the mutant with 3.0 kb deleted from the *Bam*HI site of p3BX54.

2.3. Transformation

L. acidophilus and *E. coli* were transformed using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA). *L. acidophilus* electrotransformation was performed as described previously [6]. Electroporation of *E. coli* was done according to the procedure described in the manufacturer's instruction manual.

2.4. DNA sequencing

The nucleotide sequence was determined by sequencing double-stranded DNA by the dideoxy-chain termination method [7] using a *Taq* Dye Deoxy Terminator Cycle Sequence kit (Applied Biosystems). The sequence data were analysed with the GENETYX software programs (SDC software Development Co. Ltd., Tokyo, Japan). The accession number of the DNA sequence reported in this study is D55703 in the DDBJ, EMBL, and GenBank nucleotide sequence data bases.

3. Results and discussion

3.1. Identification of the replication region of pLA103

During the course of plasmid vector construction, the region required for replication of the pLA103 plasmid was shown to be located within a 5.4-kb *Bam*HI–*Xba*I fragment. To delineate the replication region, the 5.4-kb *Bam*HI–*Xba*I fragment was cloned into *E. coli* plasmid pVC819 [6], which has an erythromycin resistance gene expressed in Gram-positive and Gram-negative bacteria, yielding the recombinant plasmid p3BX54 (Fig. 1). Derivatives of p3BX54 were made with modifications of the pLA103 portion by deletion using the restriction enzymes or by insertion of a few base pairs. The constructed plasmids were introduced into *L. acidophilus* TK1-5 (pLA103-cured mutant) [6] by means of electroporation and their replication abilities were analysed. Fig. 2 shows that the *Bam*HI–*Bgl*II fragment is obviously dispensable for plasmid replication (p3BX26). Similarly, the deletion of the *Hin*dIII–*Xba*I fragment did not affect the replication function (p3BH14). On the other hand, both deletion of the

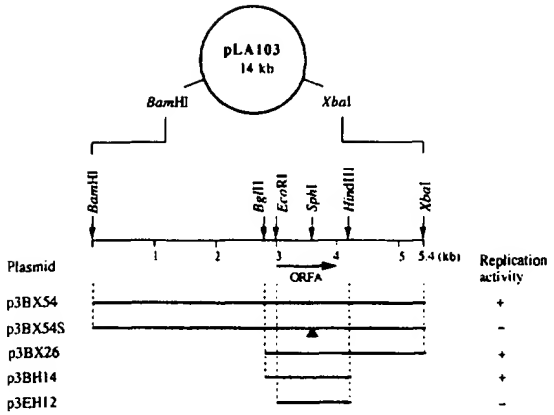

Fig. 2. The structure and replication activity of the deletion fragments of the *Bam*HI–*Xba*I fragment of pLA103. The arrow indicates ORFA found in the sequence data. The lines below the map show subcloned fragments in the indicated plasmids. The deletions were made by restriction enzymes, and the modification by insertion of a few base pairs (▲) was done using the Klenow fragment after digestion with the *Sph*I site. The replication ability of each plasmid in the *Lactobacillus* host is shown at the right: + and − indicate ability to either autonomously replicate or not, respectively.

*Bgl*II–*Eco*RI fragment (p3EH12) and modification at the *Sph*I site (p3BX54S) abolished the plasmid replication. These observations indicate that the replication determinant is localized in the 1.4-kb *Bgl*II–*Hin*dIII region.

3.2. Sequence analysis of the replication region of pLA103

The nucleotides of the *Bgl*II–*Hin*dIII fragment were sequenced (data not shown). As expected, one large open reading frame was observed. This 846-bp open reading frame, OrfA, would encode a 282-amino acid peptide with a predicted molecular mass of 33.1 kDa. Upstream of the ATG initiation codon a potential ribosome binding site (GGAGG) and a putative promoter, showing considerable similarity to the typical prokaryotic promoter sequence [8,9], are found (data not shown). A 13-bp inverted repeat sequence that can form a stem–loop structure is present 155 bp downstream of the OrfA stop codon (data not shown). The OrfA-encoded protein shared 41.2% amino acid homology with the replication protein of a 8.7-kb cryptic plasmid from *Pediococcus halophilus* ATCC 33315 (Fig. 3). These results suggest that the OrfA-encoded protein is implicated in pLA103 replication.

3.3. Analysis of the repeat sequences and their relationship with OrfA

Generally, the region necessary and sufficient for replication of plasmids contains two important components: an origin sequence (*ori*) and a *rep* gene, encoding a protein essential for the initiation of

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pLA103 (ORFA)   1   MANEIVKYENQLNSVSFRHFNARELNLFFSIVSRMRDKGTEKVSFTFNELHELSRY-SDH
                    *.**.***. .**....*.*.. *.*****.********...*.***..*.*** * ..
P.H (RepA)      1   MSNELVKYDPELNTIPLRRFTPVEMNLFFSVVSRMRDKGDDTVRFTFDQLKELSAYKPTA

                60  GERLVKDLEGVYTKMQNLNM--WYDDGNIIEHWVLFPGFQIN-RKETT-VTVSINPELKS
                    ..*...*....* *. .*..  . .**   * .*.*. *.*. . *.. *.. * *.  .
                61  NNRFIDDIQSTYQKILGLRFGSRSKDGLDREMFVMFTRFEIKGSAEVPYVDIQIYPKALK

                116 VLNQLSNWTRFSLEQFASLKSTYSKTLFRLLKQYRTVGKRNFSMQEFRNLLDIPKSY--S
                    .**.*..*.*..*..* .***.*.**.******.**.*   **. .* .*****.**  .
                121 LLNNLESWVRYALAEFRDLKSSYAKTMFRLLKQFRTTGYAYFSKSDFFELLDIPQSYWNK

                174 VSDIDKKVMTPFKKELAGIFYGLSIR-KLRKGRGGKIVGYTFTWKSERKNANDFTNGQAL
                     ......*. *...**. .* **.** * ****  ..**.******.***.**..**
                181 PANVESRVIQPIREELTPLFRGLTIRKKYGKGRGKPVIGYSFTWKSEKKNADDFSQGQFQ

                233 SNSTRKRQNKRNEPMPDYSKPKREITDADRNKL--AEQLELLQALGNHDKNK
                    ... .  . ..*. ... .* *.*.... .* .*. .* .  ..***.
                241 DERQKLFNIQHNGELTEQEK-WRAIDKVKELTLGSTEKQALADKQAEHDKKIRDQARKET
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Fig. 3. Amino acid sequence comparison of the pLA103 ORFA-encoded protein with a replication protein (RepA) of a plasmid from *Pediococcus halophilus* ATCC 33315 (P.H. EMBL accession number X75607). Identical and functionally related amino acids are indicated by an asterisk and a dot, respectively.

replication. The typical structural features of *ori* include the presence of clusters of tandem repeat sequences approximately 20 bp long [10]. The upstream region of the OrfA also contains a conspicuous sequence of 22-bp direct repeats (data not shown). To investigate whether this repeat sequence region contained *ori* sufficient to drive replication of pLA103, plasmids p3BXORI and p3BXORF were constructed (Fig. 1) and transformed into the parent *L. acidophilus* TK8912 harboring pLA103. Plasmid p3BXORI consisted of the *Bam*HI–*Sph*I fragment of pLA103, which was big enough to contain the repeat sequence region but not OrfA, and plasmid p3BXORF contained OrfA but not the repeat sequence region. Plasmid p3BXORI but not p3BXORF transformed strain TK8912 to erythromycin resistance, indicating that the repeat sequence region contains a *cis*-acting element essential for replication when provided with OrfA *in trans*. Therefore, it is conceivable that this repeat sequence region acts as the replication origin in the *Lactobacillus* host.

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